II. REMARKS

A. State of the Claims

Claims 24-45 and 61 were pending at the time of the Action, with claims 41 and 43 having been withdrawn from consideration as being directed to a non-elected species. Claims 24, 26, and 44 have been amended in the Amendment submitted herewith. No new matter has been added by these amendments. Specifically, support for the amendment to claim 24 may be found in the specification at page 6, lines 21-24, and in originally filed claim 1. Support for the amendment to claim 26 may be found throughout the specification. Claim 44 has been amended to correct a minor typographical error.

Therefore, upon entry of the amendment submitted herewith, claims 24-45 and 61 are currently pending, with claims 24-40, 42, 44-45 and 61, currently under examination.

B. Applicants' Claim for Priority is Established.

Applicants submit herewith a certified English translation of the Austrian priority document, Austrian Patent Application No. A1799/1998, as filed on October 28, 1998 (copy attached as Appendix A). Coupled with the certified copy of this Austrian application, which was filed in response to the Office Action dated December 23, 2003, the filing of this certified translation establishes Applicants' claim to the October 28, 1998, priority date.

C. The Rejections Under 35 U.S.C. § 112, Second Paragraph, are Overcome.

The Action continues to reject claim 30, based upon the use of the term "equal markers." Applicants simply mean this term to denote that all of the marker molecules in the sample are the same, *i.e.*, that the markers are "equal" in the sense of structural identity and wavelength-specific identity. This is supported in the specification at page 23, line 17, to page 24, line 5, where it is

taught that, in the context of the invention, one can use the claimed arrangement in cases where there is only one type of marker used or in cases where two or more types of marker molecules are used.

The Action rejects claim 25, as being indefinite for failing to further limit claim 24. Applicants would point out that current claim 25 recites that the "arrangement as set forth in claim 24" is "further defined as comprising biological cells in the sample holder." As such, current claim 25 requires that biological cells be present in the sample holder, while claim 24 only requires that the arrangement have a sample holder. Therefore, claim 25 further limits claim 24, and this rejection is overcome.

D.   The Rejection Under 35 U.S.C. §112, First Paragraph, is Overcome.

The Action raises a new matter rejection to claim 24 under 35 U.S.C. § 112, first paragraph. The basis for this rejection was Applicants removal of the phrase "for large area fluorescent excitation" in the Amendment submitted with the Response to the Official Action dated October 23, 2003.

Applicants respectfully point out that current claim 24 is directed to an apparatus comprising "a source of light adapted for large-area fluorescent excitation." Therefore, the rejection under 35 U.S.C. §112, first paragraph is overcome, as this claim language is supported throughout the specification.

Applicants would also point out that the phrase "large-area fluorescent excitation" is not indefinite under 35 U.S.C. §112, second paragraph. When read in the context of the specification, for example page 6, line 15, to page 7, line 21, those of skill will understand that the term "large-area fluorescent excitation" is used to distinguish and delimit the present invention, which relates to an arrangement for the practice of a "wide-field microscopy"

technique, from arrangements for the practice of "confocal microscopy" techniques. Those of skill will understand the differences between confocal and wide-field imaging. For example, these differences are extensively described in "Comparison of wide-field/Deconvolution and Confocal Microscopy for 3D Imaging" Peter J. Shaw; in *Handbook of Biological Confocal Microscopy*, 1995 (Appendix B). At page 379 of Shaw, it is stated:

> "Laser-scanning confocal and wide-field (WF) microscopy differ markedly in the way the fluorochrome molecules in the specimen are excited. In WF microscopy, each plane of the specimen is evenly illuminated while its image is recorded. In a scanning confocal microscope, the illuminating beam rapidly traverses the specimen, giving very high light intensity at the center of the focal spot and rapidly decreasing intensity over a broad region above and below this spot. The instantaneous light distribution is given approximately by the form of the WF-PSF (although the focused laser beam has a somewhat different detailed distribution). The very high light intensity can easily saturate the fluorochromes at the center of the focal spot, and the need to avoid this, in turn, limits the excitation intensity that can be used effectively. When the laser light intensity is reduced enough to avoid saturation, the amount of emitted light recorded is very small (10-20 photons/pixel) for most fluorescent biological specimens, and it is necessary to sum the light from many scans. Thus, each part of the specimen is illuminated by a succession of high-intensity pulses of light.

In view of the above, the rejection to claim 24 under 35 U.S.C. §112, first paragraph, has been overcome.

E.   The Rejections Under 35 U.S.C. § 102, are Overcome.

1.   The Rejections Over Steyer *et al.* and Eriksson *et al.* are Overcome.

Together with this response, Applicant is filing a certified English translation of the Austrian priority document to complement the previously filed certified copy of the Austrian priority document. Therefore the October 28, 1998, date of priority of the instant application is perfected. As such, Steyer may not be considered as § 102 (a) prior art since it was published on April 1999, a date after October 28, 1998. Likewise, Eriksson *et al.*, at best, as agreed by the

Examiner, is entitled only to a § 102(e) date of February 5, 1999. Therefore, Eriksson *et al.* is not prior art to the present application.

In view of the above, only the new rejections to the claims over Sharonov *et al.* must be addressed substantively. However, by determining to simply remove Steyer *et al.* and Eriksson *et al.* as prior art, Applicants in no way acquiesce to any position that any of the substance of those applications would anticipate or render obvious any of the current claims.

2. Sharonov *et al.* Does Not Anticipate the Instant Claims.

The Action enters a new rejection to claims 24-28, 31-34, and 61, stating that those claims are anticipated by the teachings of Sharonov *et al.*, which, according to the Action, discloses "an apparatus for **confocal** spectral imaging analysis." (Action, page 15, emphasis added.) Applicants traverse this rejection.

Present claim 24 is directed to:

An arrangement adapted to visualize molecules, movements of molecules, interactions between molecules, and molecular processes in a sample during use, by using a single dye tracing (SDT) method, said arrangement comprising:

at least one source of light adapted for large-area fluorescent excitation, via single or multiple photon absorption, of marker molecules in said sample during use;

a sample holder;

a detection and analysis system comprising a charged coupled device (CCD) camera, wherein at least one of the sample holder and the detection and analysis system is movable laterally, relative to the other during use; and

a control unit adapted to coordinate and synchronize illumination times and lateral movement between said sample holder and said detection and analysis system during use.

As discussed above, the instant claims are directed to arrangements that are adapted for wide-field scanning microscopy, as opposed to the confocal microscopy described in Sharonov *et al.* This distinction is made clear by the language of the current claims reading "at least one source of light adapted for large-area fluorescent excitation, via single or multiple photon absorption, of marker molecules in said sample during use." As such, Sharonov *et al.* cannot anticipate the present claims because it does not teach "at least one source of light adapted for large-area fluorescent excitation, via single or multiple photon absorption, of marker molecules in said sample during use."

Additionally, it would be clear to one of skill reading Sharonov *et al.* that the apparatus described therein is not useful to "visualize molecules, movements of molecules, interactions between molecules, and molecular processes," as claimed in the present claims. Sharonov *et al.* teaches that its apparatus splits the fluorescent light with a "single grating (G) spectrograph." (Number 7 in Fig. 2 on page 42 of Sharonov *et al.*). Such splitting indicates to one of skill that only a few photons per CCD-pixel could be collected using the Sharonov *et al.* device, and that, therefore, the sensitivity of the Sharonov *et al.* system is not sufficient to "visualize molecules, movements of molecules, interactions between molecules, and molecular processes."

In view of the above, Sharonov *et al.* does not anticipate any of the instant claims.

F.   Entry of Non-elected Species Is Requested.

In view of the forgoing arguments, all the present claims are in condition for allowance. Thus, all species contained in the dependent claims withdrawn by the examiner (claims 41 and 43) should be reentered into the case and allowed. Applicant respectfully requests that all such dependent claims be considered and allowed.

G. A Supplemental Information Disclosure Statement is Filed Herewith.

Applicant files herewith a Supplemental Information Disclosure Statement (SIDS), along with the necessary fees and a PTO Form 1449. This SIDS lists three references on the Form 1449 (C6, C14, and C18) and copies of these three references are enclosed with the SIDS.

References C6 and C14 were submitted with the original Information Disclosure Statement filed in this case, and were listed on the Form 1449 forwarded with that IDS. However, these references were not initialed as considered on the copy of the original form 1449 returned by the Examiner with the first substantive official action. Instead, the citations of these references were lined through on the Form 1449, with the handwritten notation "NO DATE" written beside the citations. Applicant would respectfully point out that, on its face reference C6 lists a publication date of December 1988, while reference C14 lists a publication date of May 1, 1997. For the convenience of the Examiner, Applicants have resubmitted these two references and have listed them again, with the publication dates, on the Form 1449 accompanying the SIDS. Applicants respectfully request that these two references be initialed as considered on the Form 1449 forwarded with the SIDS.
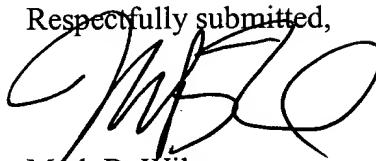
Reference C18 is a copy of the Shaw reference cited above. Applicants respectfully request that the Examiner review this reference and initial beside its citation on the Form 1449.

H. Conclusion

Applicants believe that this response places all of the pending claims in condition for allowance, and favorable reconsideration is respectfully requested.

Should the Examiner have any questions or comments, he is respectfully requested to contact Applicants' representative at (512) 536-3035.

Respectfully submitted,

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Date: July 21, 2004

VERIFICATION

I, Felicia Marchardt, of Riemergasse 14, A-1010 Vienna, Austria, do hereby declare

that I am conversant with the German and English languages and that to the best of

my knowledge and belief the following is a true and correct translation made by me of

the accompanying Official Copy of the Austrian Patent Office relating to Austrian

Patent Application No. A1799/1998.

Felicia Marchardt

Signed this 16th day of July 2004

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It is herewith certified by the Austrian Patent Office that

Prof. Dr. Hansgeorg SCHINDLER
at A-4040 Linz, Aubergstraße 43
(Upper Austria),
INNOVATIONS AGENTUR Gesellschaft m.b.H.
at A-1020 Vienna, Taborstraße 10,
on **28 October 1998**

filed a patent application relating to

"Arrangement for Visualising Molecules"

and that the specification and drawings herewith annexed are identical with the specification and drawings as originally filed together with this patent application.

Austrian Patent Office
Vienna, 16 January 2004
The President:

(Seal: Austrian Patent Office)
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R 34588

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73   Proprietor of   SCHINDLER, Hansgeorg, Prof. Dr.
            Patent: Linz (AT) and
                              INNOVATIONS AGENTUR Gesellschaft m.b.H.
                              Vienna (AT)
54   Subject: Arrangement for Visualising Molecules

61   Addition to Patent No.

67   Conversion from Utility Model

62   Division from:

22 21   Filed on: Oct. 28, 1998

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72   Inventor(s):

60   Dependence:

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tion for judging the patentability:

Form PA 31. Cover page of specification.-5000.-Z1.3090/Präs.94

The invention relates to a method for visualising molecules, interactions between molecules and molecular processes in a sample by using the single dye labeling method, as well as arrangements for carrying out such methods.

The object of highly sensitive detection systems is the observation on the level of individual atoms or molecules, respectively. This has first been made possible by the invention of the "Scanning Probe"-microscopy methods (EP 0 027 517-B1; Binnig et al., Phys. Rev. Lett. 56 (1986), pp. 930-933; Drake et al., Science 243 (1989), pp. 1586-1589). Yet, the detection of single molecules has also been made possible by optical methods. The effective conversion of light by fluorescent molecules also allowed for the detection of individual fluorophores in liquids by confocal fluorescence microscopy as well as for effecting a high resolution spectroscopy of single dye molecules at low temperatures.

The first real imaging of single dye molecules by optical means was achieved by near field optical scanning microscopy (Betzig et al., Science 262 (1993), 1422-1425). With this method, a spatial resolution of about 14 nm was achieved, which is far below the optical diffraction limit, yet application of this method is limited to immobile objects.

Furthermore, it has been possible to image single fluorescence-labeled myosin molecules on immobilised actin filaments by conventional microscopy and illumination times of seconds (Funatsu et al., Nature 374 (1995), pp. 555-559). This method is limited to observations in the immediate proximity of the substrate surface (distance of up to about 100 nm).

For allowing biological systems to be analyzed in their complete extent and for their natural function and for their physiological mode of action, visualization of individual fluorophores in complex systems and in movement is required. So far, the movement of single dye molecules has merely been illustrated for fluorescence-labeled lipids in an artificial lipid membrane system (Schmidt et al., PNAS 93 (1996), pp. 2926-2929). The methodology used for this has generally been termed "single dye tracing" or "single dye tracking" (SDT) method, since with this it is possible to trace the path of a single fluorescence-labeled molecule exactly and (as a single molecule) stoichiometrically without requiring an interaction (amplification) with

other components (e.g. by binding, spatial close relationship etc.) for signal emission.

The detection of single dye labeled molecules in cellular systems which would be required for a study of molecules or interactions between molecules in live systems is, however, not possible with the methods described. On the one hand, this is due to the fact that, in contrast to flat (planar) artificial lipid membranes, live cells are three-dimensional, and therefore the person skilled in the art would have expected considerable problems even at focussing, and, on the other hand, to the fact that cells always have a certain autofluorescence which may interfere with the fluorescence microscopy-visualizing procedure proper. Moreoever, it has been considered impossible so far to analyze a plurality of such cellular systems with a suitable detection and analyzing method so rapidly that both the resolution in the single-molecular range is maintained and also molecular movements of the molecules to be detected can be observed.

Primarily the pharmaceutical industry is more and more interested in methods with which a high throughput screening (HTS) of a large number of possible test molecules is possible. Particularly for HTS methods, however, the hitherto described methods for SDT are not suitable.

Thus, the object of the present invention consists in modifying the SDT method such that screening, in particular HTS, is made feasible therewith.

Moreover, an SDT method is to be provided by which molecular processes, preferably also in cellular systems, can be pursued in their real space-time dimension, wherein information on colocalization of molecules as well as on the stoichiometry of molecular associates and conformations of the molecules are also to be obtained.

According to the invention, this object is achieved by an arrangement for visualising molecules, their movements, and interactions between molecules, and molecular processes in a sample by using the SDT method, which arrangement comprises a sample holding means with the sample, and a highly-sensitive detection and analysis system with a charged coupled device (CCD) camera, the sample and/or the detection and analysis system being continuously shiftable relative to each other during the measuring process.

In the system according to the invention, "dyed" single molecules (e.g. fluorescence-labeled biomolecules) of a sample, in particular of a biological sample which is provided on a sample holding means, can be imaged on a pixel array of the CCD camera by the highly sensitive detection and analysis system, it being possible to continuously and constantly shift the sample and/or the detection and analysis system relative to each other, wherein (for this relative shifting) the frame shift of the CCD camera may be used so that the signals (e.g. the fluorescence photons) of each single molecule, after conversion into electrons ("counts") will be collected in the same pixels until the single molecule signal (number of "counts") exceeds a certain minimum signal/noise ratio (which ensures the significance of the measurement).

With the arrangement according to the invention a decisive progress has been achieved over the afore-mentioned methods for detecting single molecules in artificial lipid membranes (Schmidt et al., Laser und Optoelektronik 29(1) (1997), pp. 56-62), in that the system used there can also be operated as HTS method with the arrangement of the invention, on account of the shifting procedure, and, therebeyond, can be simply used on complete biological cells. By enlarging the highly sensitive detection and analysis system with a scanning system, suprisingly, a constant single molecule sensitivity could be maintained in a simple manner (since each CCD camera in principle has a frame shift (the shifting and readout speed from line to line of the pixel array of the camera)), with a maximised throughput rate, and fluorophores on or in complete cells could be imaged within a very short period of time (approximately in 120 ms).

The high-resolution detection and analysis system according to the invention must be suitable for imaging the sample on the sample holding means insofar as it must have a pixel array image of the sample with a localization of individual molecules of at least 50 to 100 nm. To this end, according to the invention, a charged coupled device camera (CCD camera) is used which hitherto has already been particularly suitable in epifluorescence microscopy. With this, precisions of the localization of less than 30 nm can be attained without any problem.

When collecting the data, the movement of the sample should be carried out constantly and continuously, since an abrupt

stopping or a high acceleration of the sample may cause the mo-
lecules to be detected in the sample, to additionally move, e.g.
on or in the cells, which could lead to longer imaging times (on
account of relaxation processes of the cell dynamics) by at
least the 10-fold, which could also induce a cell response, and
thus could lead to a falsification of the biological processes
to be observed. Usually, stepper motors are used for this, which
ensure a smoothened mode of movement by a rapid sequence of
movement steps. "Constant" and "continuous" within the scope of
the present invention means that there is no extended stopping
of the sample during the measurement process (or a measurement
in the at-rest state, respectively), but that the sample (or the
sample holding means, respectively) is always moved relative to
the detection and analysis system.

Preferably, the movement of the sample is controlled dir-
ectly by the detection and analysis system in the x-y direction,
it being possible to adapt such controlling to the respective
characteristics of the detection and analysis system. If a CCD
camera is used in the detection and analysis system, the relat-
ive shifting can be triggered directly by the frame shift char-
acteristic of the CCD camera. When a certain area on the sample
holding means is illuminated, which area is being imaged on the
entire pixel array used, the sample is continuously shifted,
and, simultaneously, the image of the sample on the pixel array
likewise is shifted line by line by continuous frame shift. In
case of an optimum adaptation of the two speeds (relative velo-
city of the movement of the sample and frame shift (line readout
speed) of the CCD camera), the information collected by a
labeled molecule of the sample while traversing the illuminated
region will be collected by practically the same pixels. Optim-
ally, the speed with which the sample is moved will be equal to
the speed of the CCD camera, divided by the magnification of the
objective.

According to the invention, mainly fluorescence dye is used
as dye, i.e. visualisation is carried out by using epifluores-
cence microscopy. According to the present state, the best res-
olutions can be attained by this method; it is, however, also
conceivable to carry out the method of the invention with other
processes (e.g. RAMAN, infrared, luminescence and enhanced RAMAN
spectroscopy as well as radioactivity), similar resolutions as

those of fluorescence technology in principle being attainable
with luminescence or enhanced RAMAN, yet above all with biolu-
minescence.

According to the invention, the use of the two-photon excit-
ation fluorescence microscopy (Sanchez et al., J. Phys. Chem.
101 (38) (1997), pp. 7020-7023) has proven particularly suit-
able, since with this method it is also possible to efficiently
circumvent the problem of the autofluorescence of many cells.

Furthermore, this allows for a practically background-free
measurement, which can also speed up HTS analysis. The two-
photon excitation fluorescence spectroscopy (or, generally,
multi-photon excitation (Yu et al., Bioimaging 4 (1996), pp.
198-207)) is particularly suitable for a three-dimensional rep-
resentation of samples, resulting in a further advantage, above
all with cellular systems.

In the embodiment with fluorescence spectroscopy, the ar-
rangement according to the invention preferably comprises:
• a laser as a precisely defined source of light, as well as
• acousto-optical switches with high specificity, by which the
  laser beam may rapidly (e.g., 10-20 nsec) be interrupted for a
  defined period of time,
• a processor which controls the switch, e.g. via a pulse pro-
  gram,
• a dichroitic mirror (which, e.g., reflects the exciting light
  upwardly towards the sample and allows the fluorescent light
  to pass downwardly from the sample (towards the analysis sys-
  tem),
• a series of suitable filters known from conventional SDT ar-
  rangements,
• a mobile sample holding means (sample holder), e.g. a pro-
  cessor-controlled x-y drive (stepper motor),
• a CCD camera by which the emitted light quantums which are
  passing the dichroitic mirror are converted into electrons and
  collected in pixels, and
• a processor which controls movement of the sample (of the
  sample holding means) by an x-y drive (stepper motor), by the
  signals from the CCD camera being used via an internal clock
  to trigger the movement.

According to the invention, it is also possible to stoi-
chiometrically label different types of molecules with a dye,

preferably a fluorescence dye, e.g. a receptor and a ligand, and to pursue both with the arrangement of the invention.

It is also possible to label at least two different types of molecules with different fluorescence dyes and to subject them to SDT analysis, wherein, in addition to the respective single fluorescence, also additional information can be obtained by determining, e.g., the Förster transfer (Mahajan et al., Nature Biotech. 16, (1998), pp. 547-552). However, it ought to be substantially emphasised that with the Förster transfer alone merely a (although highly selective) qualitative, yet not a quantitative information is possible, since this effect is highly dependent on the distance of the fluorophores (with $1/r^6$).

If cellular systems are to be assayed according to the invention, it is preferably started with cells of low autofluorescence, there being various cell types which have little autofluorescence from the beginning (such as, e.g., mast cells or smooth muscle cells). Unfortunately, however, it is just the expression cells which, as a rule, are highly fluorescent, and therefore these or other cell types having autofluorescence must be provided in a low-fluorescent state by selected growing conditions or sample processing so that their autofluorescence will be brought to below a certain interfering level. When using two-photon excitation of fluorescence, this problem, however, does not occur from the very beginning, as has been mentioned before.

With the arrangement according to the invention, carrying out a visualising method for single, e.g. biologically active, molecules is possible as a high throughput screening of biological units on the basis of the observation of single molecules (fluorophores).

High throughput screening (HTS) generally describes the search for certain "units" among a very large number of similar "units" (e.g. in a molecule library and a partial molecule library prepared by combinatorial chemistry). Such problems are encountered in many fields, both in basic bio-scientific research and also in the medically-pharmaceutically oriented industrial research and development. "Units", according to the invention, may be biological cells, yet also individual molecules or types of molecules, high throughput screening e.g. being possible for detecting rarely occurring cells having a certain genetic defect. Besides its usefulness in connection with questions of

cellular biology and pathology, high throughput screening is important in molecular biology. Thus, the arrangement according to the invention may, e.g., be used to find single DNA or c-DNA molecules in a sample comprising many DNA molecules. In biochemistry, the separation of macromolecules having certain properties, e.g. with respect to ligand binding or state of phosphorylation in or on cells, is a basic requirement which can be dealt with according to the invention. The pharmaceutical industry needs high throughput screening both for selecting certain active agents and also for analyzing their activity on biological cells. Each person skilled in the art will know what belongs to HTS methods or which materials can be used therefor (e.g. molecule libraries prepared by combinatorial chemistry or genomic-combinatorial libraries) (cf., e.g., "High Throughput Screening", John P. Devlin (Ed.) Marcel Dekker Inc. (1997)).

For a specific labeling of certain "units", according to the invention mostly the natural principles of the structurally-specific molecular recognition are employed, such as the binding of antibodies or, generally, of ligands to receptor molecules. The preferred use according to the invention of fluorescent ligands, such as antibodies with bound fluorescence molecules, allows for a both sensitive and selective detection of units with receptors for the fluorescence-labeled ligands. As an alternative to fluorescent ligands, fluorescent groups can be inserted in protein sequences and coexpressed (e.g. the "green fluorescence protein" (GFP) or variants thereof ("blue fluorescence protein" - BFP)).

According to the invention, with the use of fluorescence, a high throughput screening with simultaneous ultimative sensitivity (i.e. clear detection of the fluorescence of individual fluorescence markers) and high throughput rate (i.e., at least 10^6 (cellular) units per inch^2 per hour) can be realised. Chemical units (e.g. biological molecules, such as receptor agonists or antagonists) may be assayed without any problem with a throughput rate of at least 10^{10} or 10^{12} units per hour per inch^2.

When using cells in a HTS method, primarily microtiter plates are suitable with which a medicament screening can be carried out on complete cells, e.g. by titrating the cells into the individual wells which contain the substances to be screened (cf. e.g. WO 98/08092). Also the use or measurement of biochips

(Nature Biotech. 16 (1998), 981-983) is possible with the system according to the invention.

If substances are identified as pharmaceutical target substances and isolated with the HTS method of the invention, which are new or for which so far a pharmaceutical activity could not be demonstrated, the present invention, in a further aspect, relates to a method for preparing a pharmaceutical composition, which comprises mixing of the substance identified and isolated according to the invention with a pharmaceutically acceptable carrier.

According to the invention, a clear detection is considered to be given if the minimum signal/noise ratio determined for single molecules is more than 3, preferably between 10 and 40, in particular between 20 and 30. If the signal/noise ratio is below a value of approximately 2 to 3, interpretation of the information content of the measurement obtained may be a problem.

A specific variant of the method according to the invention is the combination with the flow cytometry technology, in which the cells are moved by a flow cytometer past the detection and analysis system. In the simplest instance, in a preferred variant of the arrangement of the invention, a flowthrough cell is provided with the sample holding means (or as the sample holding means itself, respectively).

As has already been mentioned, the arrangement according to the invention is particularly suitable for the analysis of samples which comprise biological cells, wherein particularly HTS methods may be carried out efficiently with the arrangement according to the invention. The spectrum of use of the arrangement of the invention is, however, also highly efficiently applicable to cell-free systems.

In the arrangement according to the invention, the continuous relative shifting between sample and the highly sensitive (high-resolution) detection and analysis system preferably is controlled by the detection and analysis system itself, in particular by the CCD camera.

Since fluorescence analysis at present yields the best analyses, the arrangement according to the invention preferably comprises an EPI fluorescence microscope. Moreover, control of the continuous relative shifting can be triggered via the frame shift of the CCD camera, control being directly effected through

the CCD camera, or in parallel by a synchronisation mechanism (e.g. location-correlated via photodiode triggering signals by using a co-transported punched tape, such as, e.g., described in Meyer et al., Biophys. J. 54 (1988), pp. 983-993).

A preferred embodiment of the present invention therefore is characterised in that the sample movement and the frame shift of the CCD camera are synchronised with each other by location-correlated signals derived from the continuous sample movement, preferably by using a punched tape moved together with the sample, and a fixed photodiode which emits a signal when passing a punched hole.

In a further aspect, the present invention relates to a method for visualising molecules, interactions between molecules, and molecular processes in a sample by using the SDT method employing an arrangement according to the invention.

The fields of application for the present invention are practically unlimited, preferred are, however, pharmacy (primarily HTS of new chemical units) as well as biochemical questions, since, due to the extremely high sensitivity of the methodology according to the invention (a single molecule can be pursued) and the exact localisation (e.g. with a precision to at least 30 nm) basically each individual molecule or molecule associate, e.g. on or in cells, can be detected and identified (optionally also isolated). Thus, the bindings of all natural ligands to a cell (hormones, primary messenger substances, etc.) or cell-cell recognition molecules with molar binding can be analyzed, also as regards the exact binding kinetics and binding conformation, as well as regards the mobility of these components within the cell or within the cell membrane (analogous to Schmidt et al., J. Phys. Chem. 99 (1995), pp. 17662-17668 (for molecule position and mobility determinations); Schütz et al., Biophys. J. 73 (1997), pp. 1-8; Schmidt et al., Anal. Chem. 68 (1996), pp. 4397-4401 (for stoichiometric determinations); Schütz et al., Optics Lett. 22 (9), pp. 651-653 (as regards conformation changes).

Furthermore, the system according to the invention is particularly well suited for analyzing and identifying or isolating, respectively, (alternative) binding partners in receptor-ligand or virus-receptor systems, wherein also potential agonists/antagonists and their action (e.g. the competitive inhibi-

tion) can be precisely analyzed. This is particularly essential when finding new chemical units (NCU) in the field of medicament screenings.

When analyzing entire cells, the focus plane may be varied; in a rapid variant, a section through the cell (preferably, the upper cell half; "lower" meaning the side facing the sample holding means) is analyzed. Thus, it is also possible to analyze complex processes in a cell, such as nucleopore-transport, the effect of pharmaceuticals with a target in the cell or secondary reactions in the cell, on single molecule level.

According to a preferred embodiment, the system of the invention may also be used to analyze three-dimensionally (3D) occurring processes in single cells, such as cells which have been pre-selected in a first area scan according to the invention. In doing so, by a continuous shift of the focus plane along the z axis, in addition to the inventive mode of procedure (sample shifting with synchronised frame shift of the CCD camera), the three-dimensional arrangement of fluorescence-labeled molecules or associates on or in the cell can be imaged, in measurement times in the range of seconds or even therebelow, with a spatial resolution close to the diffraction limit. Compared to the hitherto only other method, the confocal scanning fluorescence microscopy, CSFM (Handbook of Biological Confocal Microscopy, ed. James B. Pawley, second edition (1995), Plenum Press, New York and London), the illustrated, above-indicated method according to the invention, firstly, is more rapid by at least a factor 1000, since simultaneously the information with equal resolution can be collected from about 1000 focus areas, whereby, secondly, it is possible for the first time to image non-static molecules or associates, respectively, in spatial-temporal arrangement in periods of time (1 s, e.g.,) which are small enough to observe diffusion processes, energy-driven movements or metabolic processes.

In a preferred embodiment, thus, the focus plane of the detection and analysis system (in particular, of the epifluorescence microscope) can be shifted along the z direction (i.e., normal to the x-y plane which is defined by the sample surface (the sample holding means)), optionally in addition to the relative movement between sample and detection and analysis system.

The invention will now be explained in more detail by way of

the following Examples and drawing figures, without, however, being restricted thereto.

Fig. 1 shows the usual configurations of units for high throughput screening;

Fig. 2 shows one possible arrangement according to the invention;

Fig. 3 shows the relative movement of the sample with frame shift;

Fig. 4 shows the screening of units on surfaces or in multi-well plates;

Fig. 5 shows the screening in a laminar flow cell;

Fig. 6 shows the relation between screening time and resolution;

Fig. 7 shows the analysis of detected units;

Fig. 8 shows the use of donor-acceptor energy transfer for highly selective screening;

Fig. 9 shows the positions of labelled molecules;

Fig. 10 shows the temporal tracing of molecule positions;

Fig. 11 shows the molecular association, co-localization, stoichiometry from signal quantization;

Fig. 12 shows the ligand binding;

Fig. 13 shows the conformation change on the single molecule;

Figs. 14-18 show the detection of individual lipid molecules in native cells;

Fig. 19 shows the microscopy of individual lipid molecules with two-photon fluorescence excitation;

Fig. 20 shows the three-dimensional imaging of a selected single cell with single fluorophore resolution.

E x a m p l e s :

E x a m p l e 1: Arrangement according to the invention, employing fluorescence microscopy

Conventionally used configurations of units for high throughput screening (HTS) are illustrated in Fig. 1, which are all employed as measurement arrangements in the method according to the invention. Usual molecule libraries, prepared by combin-atorial chemistry, are assembled on small (0.2 to 0.4 mm) poly-

mer beads each carrying a single molecule species (cf., e.g., Devlin (1997), pp. 147-274). The measurement arrangement according to the embodiment described above starts from a conventional fluorescence microscope (Fig. 2) by means of which fluorophores present on the substrate surface in the illuminated area ($\sim 100 \mu m^2$) could be individually detected and their movement could be followed, with a signal-to-noise ratio of ~ 30 for single fluorophores (published in Proc. Natl. Acad. Sci., USA (1996) 93: 2926-2929). A Zeiss microscope (Axiovert 135-TV) having a x100 objective (Neofluar; numeric aperture = 1.3, Zeiss) was used. For the fluorescence excitation, the laser light of the 514 nm line of an argon$^+$ laser (Innova 306, coherent), which was operated in TEM_{00} mode, was coupled through an acousto-optical modulator (1205C-1; Isomet) in the epiport of the microscope. A $\lambda/4$ plate delivered circular-polarised excitation light. By using a defocussing lens (f = 100 mm) in front of the dichroic mirror (515DRLEXT02; Omega), the Gaussian excitation profile was set to 6.1 ± 0.8 μm full width at half maximum (FWHM) and 57 ± 15 kW/cm^2 mean excitation intensity. The illumination time for each pixel array image was 5 ms. After long-pass filtering (570DF70 Omega and OG550-3 Schott), the fluorescence was detected by a liquid-nitrogen-cooled CCD camera (AT200, 4 counts/pixel read-out noise; Photometrix), equipped with a TH512B chip (512 x 512 pixel, 27 μm^2 pixel size; Tektronix). The point-transfer function of the microscope was described by a two-dimensional Gaussian intensity distribution with a width of 0.42 μm FWHM, as was found by determining images of 30 nm fluorescent beads (Molecular Probes). The diffraction-limited area thus was 0.14 μm^2. With 0.48 ± 0.08 μm FWHM, the width of intensity profiles for single molecules was larger than the point-transfer function of the microscope, the additional broadening having been caused by molecular diffusion. The CCD was used as a memory means, with 12 consecutive images of 40 x 40 pixels being captured, wherein up to 140 pixel arrays could be imaged per second, due to CCD frame shift. This frame shift is used according to the invention for continuous movement of a sample holding means.

According to the present invention, this measurement principle can be applied to biological samples with fluorescent ligands in configurations as illustrated in Fig. 1. According to the apparatus by which the invention has been realised, sample

screening is enabled which has a constant single fluorophore sensitivity with maximised throughput rate. The basic idea is once more explained in Fig. 3. At constant illumination of an area which is imaged on the total pixel array used, the sample is continuously shifted and, simultaneously, the image of the sample on the pixel array by continuous frame shift, line per line. With as precise a coordination of the two velocities (v (sample) = v(CCD)/magnification of the objective) as possible, the fluorescence collected from a fluorophore of the sample will be collected during traverse of the illuminated area by practically the same pixels.

In Figs. 4 and 5, the cumulating image of a fluorophore up to reaching the read-out side of the pixel array is outlined for screening configurations according to Fig. 1. Optimisation of the numerous apparatus variables and parameters is possible for the skilled artisan in analogy to the known methods; the ratio between resolution and measuring time is shown in Fig. 6: For typical characteristics of obtainable CCD cameras, sources of light and objectives, the measuring time for the screening of an area of 1 inch^2 was calculated, as a function of the resolution. Basically, there is a clear-cut region of the optimum relationship between measuring time and resolution, which in the example chosen is in the range of measuring times of from 20 to 90 min for 1 inch^2 (6.45 cm^2) sample area, at a resolution of from 3-0.5 μm. The working point on this curve is adjusted by binning (right-hand scale in Fig. 6). To this end, the information of neighbouring pixels is combined (e.g. of b x b pixels), whereby the resolution decreases, at increasing maximum velocity v (frame shift) of the CCD camera and slightly increasing sensitivity. The latter is based on the fact that the noise merely is due to read-out noise, and thus is equal in amount for the read-out of the counts in a single pixel as for the read-out of the counts in b x b pixels. The imaging quality of individual fluorophores thus is substantially maintained during screening (in the example according to Fig. 6, 500 counts per fluorophore are collected at 5 counts of read-out noise).

By the continuous sample movement, the waiting time is minimised which is necessary at discontinuous sample movement, due to the movement forming in the sample when the velocity is changed. Merely after termination of a line scan, the sample has

to be returned and shifted by the width of the illuminated area, so as to collect the next line scan (in the example of Fig. 6, a waiting time of 5 s was allowed therefor).

The inventive combination of ultimative sensitivity and comparatively very rapid sample throughput opens up new fields of application. With a screening time of ~30 min of a sample of typical size, a time range has been attained which allows for a screening under generally constant conditions of the samples. Samples with a correspondingly short life time can be assayed, and the detected units can be further used or analyzed. This, moreover, allows for the use of a wide range of fluorescence ligands with appropriately rapid dissociation rates (e.g., weakly binding antibodies). The simultaneous single fluorophore sensitivity basically enlarges the field of application to situations in which labeled sites per unit sought are to be expected in low numbers (down to a single site, such as when finding a mutation in a DNA sample).

According to the invention, the rapid and sensitive screening described can be further combined with a high selectivity and specificity. To this end, selective excitation of the fluorescence markers by two-photon absorption is used, whereby the fluorescence collected almost entirely comes merely from the thus-excited fluorescence markers in the focus area. In a further mode of action, two fluorescence-labeled ligands are used simultaneously, which both have neighbouring binding sites on the target structure. This may, e.g., be a natural ligand of a receptor merely occurring in the unit sought, together with an antibody which binds to the receptor molecule in the vicinity of the ligand. As outlined in Fig. 8, in case of a selective excitation of one of the two fluorophores (donor) and collection of the fluorescence (by appropriate optical filters) of only the second fluorophore (acceptor), merely the fluorescence formed by the transfer of energy from the donor to the acceptor will be detected. For this purpose, both fluorophores have to be in the immediate vicinity (distance $\leq$ 8 nm). In this way, ligand pairs specifically bound to receptors become detectable individually and highly selectively (with still a high signal/noise ratio). Besides increasing the selectivity of fluorescence by transfer of energy, the specificity of the signal can be increased by illumination in total reflection (cf. Fig. 3 below). Thus, only

those fluorophores are excited which are located in a range of approximately 100 nm from the substrate surface (exponentially fading light intensity). The detection sensitivity also reaches single fluorophores. This type of illumination shall complement the invention by enabling the use of high throughput screening for units (mainly cells) having a high autofluorescence.

According to the invention, immediately after the detection of sought units by screening, the same apparatus allows for a detailed analysis of these units. This may either be carried out directly in the screening sample, or after transfer of a unit into an analysis cell. Fig. 7 shows this on the example of a biological cell.

The analysis cell allows for single molecule microscopy in a region of the biological cell which is freely accessible to an exchangeable buffer solution and active substance. Furthermore, the cell is practically tightly bound electrically to the substrate so that the highly sensitive fluorescence microscopy can be combined with electrophysiology, e.g. for observing single ion channels, electrically and optically.

Figs. 9 - 13 outline five basic types of information which become possible by single molecule microscopy on transferred units. In this connection, binning is employed to adapt the temporal and lateral resolution to the desired information. The sample is not moved, and short (ms), periodically repeated illumination is employed. This allows for each illumination to detect the positions of sufficiently far removed single fluorophores (Fig. 9) and to follow them temporally (Fig. 10). Thus it can be decided whether a labeled receptor is mobile, restrictedly mobile, or immobile, diffuses freely or has limited diffusion or is self-associated, co-associated with other components, or transiently clustered. Also the distribution over the (cell) surface can be made visible. The high signal and the high signal/noise ratio S/N (approximately 150 counts and S/N = 30 for 5 ms of illumination) allows for the allocation of observed signals to the number of co-localised fluorophores. This opens the field for numerous mechanistic studies relating to the association, co-localisation and stoichiometry of associated components, outlined in Fig. 11 for dimerization of a membrane component. Also the ligand binding becomes analysable on a single-molecular level (Fig. 12), including the stoichiometry of

the ligand binding, as well as allosteric and cooperative effects in ligand binding.

Special ligands (whose fluorophore points into a fixed direction after binding to the receptor) can be employed for single-molecular detection of conformational changes. A slight rotation of the fluorophore with a structural change of the receptor suffices to detect the conformational change via the intensity change of its fluorescence signal, as is outlined in Fig. 13. For this, both linearly polarised light of different directions of polarisation and circularly polarised light are used.

The inventive continuous imaging of the fluorophores in the sample by synchronous movement of the sample and CCD frame shift (according to Figs. 3 to 6) has neither been described nor suggested in the prior art relating to single fluorophore imaging, since there, only static images have been captured in immobile samples. With the system according to the invention, in addition to the ultimative optical resolution and sensitivity of the time-resolved detection of single molecules (e.g., receptors on cells), a considerable screening speed has become possible which is at least 1000 times more rapid than in the alternative methods of confocal microscopy, with simultaneous observation of an ensemble of molecules which is not possible by confocal microscopy.

E x a m p l e   2 : Detection of fluorescence-labeled lipid molecules in the plasma membrane of native smooth muscle cells

Methodology: smooth muscle cell, HASM: human aorta smooth muscle, stable cell line of wild type, are allowed to grow on a cover glass and subjected to microscopy in PBS buffer. Incorporation of DMPE-Cy5 (dimiristoyl-phosphatidyl-ethanolamine with Cy5 (from AMERSHAM) bound as dye molecule) is effected via lipid vesicle (POPC: palmitoyl-oleoyl-phosphatidylcholine, from AVANTI). Each 1000th lipid in the vesicles was a DMPE-Cy5 (mean: 10 DMPE-Cy5 per vesicle). Addition of these vesicles via the flowthrough cell to the HASM cells in the microscope (50 µg/ml vesicle, incubation for 10 min, then washed out with PBS buffer) leads to DMPE-Cy5 individually incorporated in the plasma membrane, via vesicle/cell membrane/lipid exchange. This process of the delivery of one DMPE-Cy5 to the plasma membrane is directly visible in Fig. 14, the vesicle (at ~ 10 DMPE-Cy5, cf. high sig-

nal) quickly diffusing along the cell membrane and one DMPE-Cy5
suddenly changing over from the vesicle into the plasma membrane
(cf. slight signal in Fig. 14), and there it can be imaged (Fig.
15). Such an exchange could not be observed previously on single
molecule level. What is essential, however, is that the intens-
ity of one fluorophore is still clearly resolved in the cell
having autofluorescence. In the present example (Figs. 14-18),
the intensity of the laser light (630 nm) was reduced such that
the effective fluorescence background of the cell was only about
5% of the signal (S/B≈15). The intensity may, however, be in-
creased at any time, so that - via the autofluorescence of the
cell - it is possible to get an orientation regarding the site
at which the measurement is being carried out. This value of
S/B≈15 is comparable with the limit value of the signal/noise ra-
tio of 25 which is given by the readout electronic equipment at
the intensity used. The illumination time was 5 ms. For a better
understanding of the peaks shown: The area shown comprises 40 x
40 pixels, and this corresponds to an object area of ~10 x 10 μm
(each pixel is 27 x 27 μm, a x 100 objective was used). The HASM
cell has a length of approximately 100 μm, a width of 15-20 μm,
and a height of 5-10 μm. The illustration is diffraction-limited,
i.e. each dot source is imaged as a Gaussian spot having a radi-
us of $\lambda/2$ = 315 nm, this corresponds to 1.2 pixels (50% of the
peak on 5 pixels). In the peak there were 152 cnts on an aver-
age.

As a matter of routine, sequences of up to 14 images were
captured (e.g. 9 images according to Fig. 16), 5 ms illumination
each, with dark intervals of between 10 to 30 ms (i.e. measuring
times of up to ~0.5 sec). These result in trajectories for the
movement of the labeled lipids in the plasma membrane (Fig. 17
shows the trajectory to the images according to Fig. 16). ~100
of such trajectories were evaluated (includes measurements on
three different cells and on different locations of the cells,
yet always on the upper side of the cells which are adhered on
the bottom of the cover glass). In the measuring time of
0.5 sec, no convection or other cell movement was observed (ex-
cept for a few erratic cell jerks). The result was impressive:
The evaluation of the trajectories is illustrated in Fig. 18:
The square of the distance (MSD = mean square displacement)
between observed molecule positions and trajectories is entered

against the respective time interval Δt. With Brown's diffusion processes, this should result in a linear connection; MSD = $4D_{lat} \cdot \Delta t$, with the diffusion constant D_{lat} for lateral movement resulting from the ascent $4D_{lat}$. At first, for short diffusion lengths, a diffusion with $D_{lat} = 0.5$ $\mu m^2/sec$ appears, a typical value for lipid diffusion in cell membranes (from ensemble measurements via FRAP : fluorescence recovery after photobleaching). For lengths of 240 nm and more, a slower effective diffusion is suddenly seen, D_{lat} (MSD > 240 nm) = 0.14 $\mu m^2/sec$. This and a closer analysis of the trajectories clearly shows that the delay originates from a diffusion barrier (reflection coefficient of 0.75; plausible: every 4th pulse leads to an overcoming of the barrier), which separates the regions of free diffusion. These barriers must yield an even pattern (otherwise the curve in Fig. 18 would not be so steep) and separate free regions with effective diameters of 240 nm. This is precisely the expected value which results from the spectrin membrane skeleton (which exists for this smooth muscle cell, spectrin 230), which mainly forms a square grid structure having a length of 200 nm, from which a mean diffusion length of 226 nm can be calculated. Fig. 18 contains a further set of data for LysoPE-Cy5 (i.e. the same molecule, yet only with a myristoyl chain), by which it is shown that this molecule diffuses approximately equally quickly as DMPE-Cy5, yet does not feel any barrier. With this, thus, a method is provided for investigating the role of the membrane skeleton at the compartment formation in the membrane (important for many functions and only very poorly characterised due to the absence of suitable measurement methods) for many cell types. These results of the test shown (the S/B ratio and the S/N may be improved 2 to 3 times by using holographic notch filters) therefore had to be considered as surprising. In principle, this indicates that any application of SDT, also as hitherto has been used on model systems, opens up new possibilities, simply due to the fact that processes can be viewed on a single-molecular basis and dynamically, which so far have been accessible merely via ensemble-mediated data. In this connection, the present proof is essential that single fluorophores on live cells (at least on these smooth muscle cells) can be viewed by microscopy clearly and with time resolution. The marker, Cy-5, may also be attached to a ligand having the same fluorescence properties.

The frame-sample-shift reduces this resolution only unsubstantially, it serves for the continous screening of complete cell cultures or cells in nanotiter plates etc. Resolution can be further improved by two-photon-excitation fluorescence microscopy. Fig. 19 shows the first realisation of a two-photon imaging of two phospholipids (PE-) with bound TMR (tetramethyl-rhodamine) as fluorescence markers in a phospholipid (POPC) membrane.

E x a m p l e   3 : Simulation of a real time image of the distribution of single fluorophores on complete cells, the spatial-temporal resolution, the position precision and the detection safety of the fluorophores

In Example 2, the observation of the single lipid diffusion in the plasma membrane of the cell i.a. was possible because the plane of the lipid movement (membrane surface) could be brought into register with the focus plane (layer with an effective thickness of 1.6 µm) to a sufficient extent, which was realised by focussing on the upper rim of the cell.

To capture movements in any direction, also transversely to the focus plane, at any location on or in the cell, as well as almost at the same time for all the fluorescence-labeled molecules, the sample movement according to the invention and frame shift is carried out in the following variant:

Methodology: The methodology and the arrangement for imaging is as in Example 2, with two substantial differences:

1.) The cell is moved at constant velocity "v(z)" in z-direction so that the focus layer continuously moves with "v(z)" through the entire cell. This is outlined in Fig. 20A, with the focus layer in red, (effective thickness 1.6 µm), a cell in green (approximate height of 8 µm), with an ensemble of randomly distributed equal fluorophores (black dots).

2.) A CCD camera of the following specifications is being used: with large, elongate pixel array and particularly rapid frame shift (e.g. a CCD camera with 2048 x 256 pixels, 7 µs shift time per line, and 2 µs/pixel readout time, conversion of 0.8 electrons/red photon, as is offered for spectroscopy by PHOTO-METRICS, e.g.).

In the exemplary embodiment (Fig. 20), 20 images are captured while the focus layer passes through the entire cell. Each image is taken with the same illumination time "t(ill)" on

the same partial area of the pixel array (gray area in Fig. 20A with 100x256 pixels), and then shifted by frame shift, as illustrated in Fig. 20A for the first and the last image. During the time required for the frame shift "t(fs)", 0.7 ms with the above-specified camera, imaging is interrupted (by interrupting the illumination or covering the camera), yet not so the movement of the cell.

The velocity "v(z)" and the illumination time per image "t(ill)" must be adjusted to each other according to the relation v(z)=dz/t(ill), with "dz" being the shifting of the cell per image captured, which is chosen to be equal to 0.4 µm in the exemplary embodiment, so that the 20 images will just cover the entire cell of 8 µm height (cf. Fig. 20A).

The time "t(ill)" is freely selectable within limits. On the one hand, "t(ill)" should be substantially longer than "t(fs)" to keep low the information loss due to the illumination pauses. On the other hand, the entire imaging time
t(total)=(t(ill)+t(fs))x 20 should not be too long (long "t(ill)"-times are advantageous to even out unspecific fluorescence) so that the molecule ensemble of the entire cell can nearly be imaged in real time. Real time imaging requires t(total)<t(mov), wherein "t(mov)" is the time which is required by the molecules imaged to move over a longitudinal extension which corresponds to the optical resolution (approximately 0.5 µm in x- and y-direction, and approximately 1.6 µm in z-direction). For diffusion of typical membrane proteins or of actively transported components, "t(mov)" is mostly below approximately 0.6 s. From this there results for the exemplary embodiment a range of 5 ms < t(ill) < 30 ms for real time imaging of the fluorophores of a cell. The entire imaging will then take 0.1 s < t(total) < 0.6 s.

Fig. 20B illustrates the calculated result of real time imaging of fluorophores on a cell membrane for t(ill)=5 ms, wherein the data and conditions measured in Example 2 were taken as a basis for single images (6 counts/pixel of autofluorescence of the cell, 152 counts/fluorophore for 5 ms illumination with an intensity averaged over the focus depth, lateral resolution of 0.5 µm, and focus depth of 1.6 µm). These data set all the parameters for calculating the 3D image for the above-described method, a rotation ellipsoid with random deviations being chosen

as the cell form (Fig. 20A shows the front view of the cell), and with randomly distributed fluorophores on the cell membrane (black dots, total of 45). Fig. 20B shows the front view of the 3D fluorescence image of the cell and of the fluorophores, produced by complete simulation of the imaging method (in the simulation, each fluorophore emits fluorescence photons corresponding to the illumination at the moment through the shifting focus layer, which are imaged in random distribution on corresponding pixels, taking into consideration the diffraction-limited imaging function of the microscope, after corresponding conversion to electrons as "counts"). The colour code chosen shows green for a low count number (autofluorescence of the cell and readout noise), and yellow, light red to dark red for an increasing number of counts. The colour code is chosen such that the light red range approximately reproduces the resolution volume (due to the collection statistics slightly inexact ellipsoid with diameters of approximately 0.5 μm in the x-y plane and 1.6 μm in z-direction), and that the dark red core reflects the precision of the positioning of individual dyes (approximately 100 nm in the x-y plane and 200 nm in z-direction). The loss of data on account of dark periods during the frame shift could be approximately taken into consideration by interpolation between the intensities of consecutive images by the fact that in the mean, each fluorophore appears in 4 images (focus layer thickness = 1.6 μm = 4 x dz, with dz = image-to-image shift = 0.4 μm).

The simulation shows that 3D cell images in real time and with a clear detection of the fluorescence-labeled molecules are possible with the method according to the invention. In principle, this 3D image of the cell can be repeated several times after a minimum time each "t(read)" which is necessary to read out the pixel arrays (approximately 1 s with the above-mentioned camera). The number of repetitions is limited by bleaching of the fluorophores. For the conditions assumed in Example 2, at least a 5-fold repetition is possible without large losses by bleachout, and an up to 15-fold repetition when using optical notch filters. In principle, the method cannot only image fluorophores on cells, as is illustrated in the exemplary embodiment, but also fluorophores in cells. To this end, the use of two-photon excitation may be advantageous, at least for studies

on cells with high or not minimised autofluorescence.

Such a sequence of real time 3D images of molecule ensembles of a cell do not only go far beyond the prior art, but in terms of quality they open up new ways for analysis of cyto-physiological processes, such as the uncovering and analysis of component organization/reorganization as an essential basis for the spatial-temporal regulation and coordination of cellular processes, or the mechanistic analysis of morphological responses of the cell to an external stimulus by, e.g., a messenger substance or by a pharmacologically active substance, or by a possible active substance identified according to the invention according to Example 1.

Claims:

1. An arrangement for visualising molecules, movements there-
of, and interactions between molecules, and molecular processes
in a sample, by using the single dye tracing (SDT) method, com-
prising a sample holding means with the sample, a highly-sensit-
ive detection and analysis system comprising a charged coupled
device (CCD) camera, the sample and/or the detection and analys-
is system being continuously shiftable relative to each other
during the measuring process.

2. An arrangement according to claim 1, characterised in that
the relative displacement between the sample and the detection
and analysis system is controlled by the detection and analysis
system, in particular the CCD camera.

3. An arrangement according to claim 1 or 2, characterised in
that it comprises an epifluorescence microscope.

4. An arrangement according to any one of claims 1 to 3, char-
acterised in that it comprises an N_2-cooled CCD camera in the de-
tection and analysis system.

5. An arrangement according to claim 4, characterised in that
the control of the relative movement is effected according to
the frame shift of the CCD camera.

6. An arrangement according to any one of claims 1 to 5, char-
acterised in that the sample comprises a molecule library pro-
duced by combinatorial chemistry.

7. An arrangement according to any one of claims 1 to 6, char-
acterised in that the sample holding means is a flowthrough
cell.

8. An arrangement according to any one of claims 1 to 7, char-
acterised in that the relative shift of the sample is directly
controlled by the frame shift of the CCD camera.

9. An arrangement according to any one of claims 1 to 7, char-

acterised in that the sample movement and the frame shift of the CCD camera are synchronised with each other by location-correlated signals derived from the continuous sample movement, preferably by using a punched tape moved together with the sample, and a fixed photodiode which emits a signal when passing a punched hole.

10. An arrangement according to any one of claims 1 to 9, characterised in that the focussing plane is shiftable along the z-direction in addition to the relative movement between the sample and the detection and analysis system.

11. An arrangement according to any one of claims 1 to 10, characterised in that it comprises a two-photon fluorescence excitation laser.

12. A method for visualising molecules, movements thereof, and interactions between molecules, and molecular processes in a sample by using an arrangement according to any one of claims 1 to 9, characterised in that the sample to be tested and located on the sample holding means is imaged by the CCD camera on a pixel array, wherein the sample and/or the detection and analysis system are shifted constantly and continuously relative to each other by using the frame shift of the CCD camera, so that the signals of each individual molecule in the sample are collected in the same pixels after conversion into electrons until the single molecule signal exceeds a certain minimum signal/noise ratio.

13. A method according to claim 12, characterised in that the movement of the sample is controlled corresponding to the frame shift of the CCD camera.

14. A method according to claim 12 or 13, characterised in that different types of molecules are labelled with a fluorescence dye.

15. A method according to any one of claims 13 to 14, characterised in that at least two different types of molecules are fluorescence-labelled with different fluorescence dyes.

16.　A method according to any one of claims 12 to 15, characterised in that the sample to be examined comprises biological cells, preferably cells with low autofluorescence.

17.　A method according to any one of claims 12 to 16, characterised in that the method is carried out as a high throughput analysis.

18.　A method according to any one of claims 12 to 17, characterised in that the interaction of a molecule library, prepared by combinatorial chemistry, with biological cells is analyzed.

19.　A method according to any one of claims 12 to 18, characterised in that the certain minimum signal/noise ratio for individual fluorophores is higher than 3, preferably between 10 and 40, in particular between 20 and 30.

20.　A method according to any one of claims 12 to 19, characterised in that at least 10^6 biological cells or at least 10^{10} molecules per inch^2 (6.45 cm^2) are analysed per hour.

A b s t r a c t :

An arrangement for visualising molecules, movements thereof, and interactions between molecules, and molecular processes in a sample, by using the single dye tracing (SDT) method is described, comprising a sample holding means with the sample, a highly-sensitive detection and analysis system comprising a charged coupled device (CCD) camera, the sample and/or the detection and analysis system being continuously shiftable relative to each other during the measuring process.

Fig. 4

FIG. 1

CONFIGURATIONS for SCREENING

★: Fluorescence marker, specifically bound to units sought
(biol. cells, DNS, c-DNS, macromolecules, proteins, etc.)

Units on substrate

Units in wells

Units in laminar flow cells
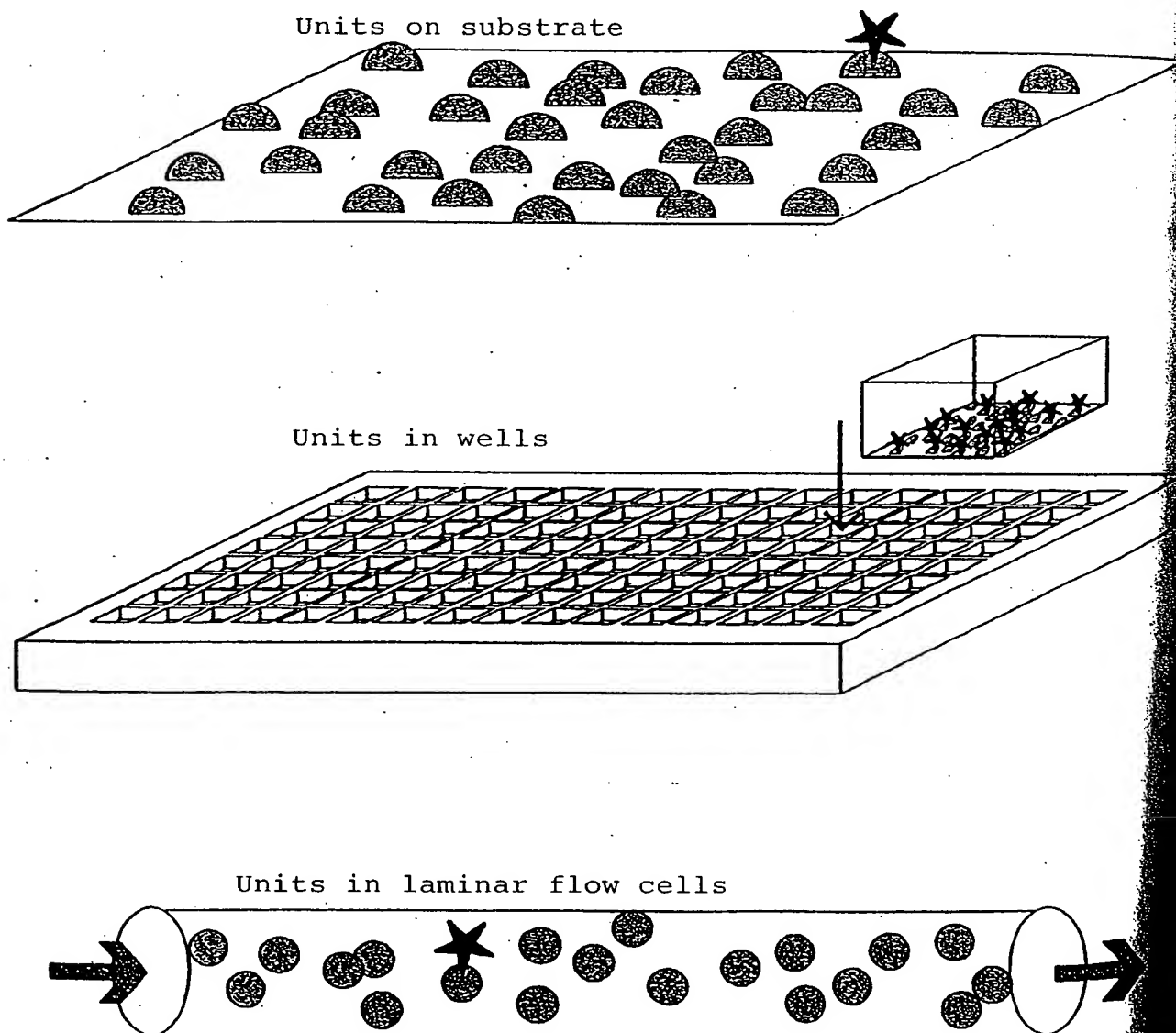
FIG. 2

TIME-RESOLVED MICROSCOPY OF

INDIVIDUAL FLUORESCENCE MOLECULES

Fluorescence molecule

Objective

Laser

Filter

CCD Camera

Image of individual
fluorophores,
5ms illumination,
signal/noise ratio
~30
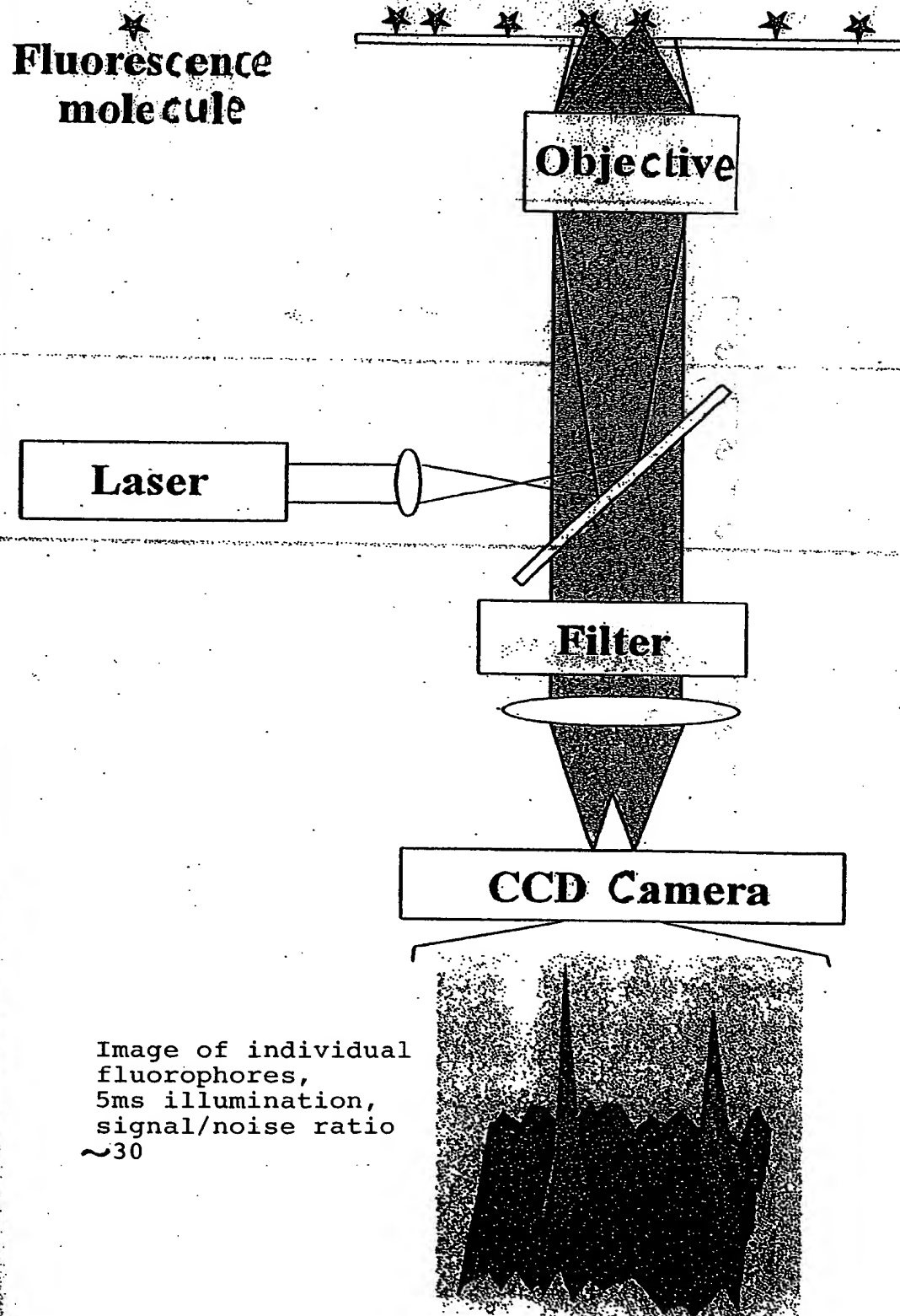
FIG. 3

USE of SINGLE FLUOROPHORE-

MICROSCOPY for SCREENING

"sampleshift" with velocity
v(sample)=v(CCD)/magnification

CCD "frameshift" with velocity   **v(CCD)**
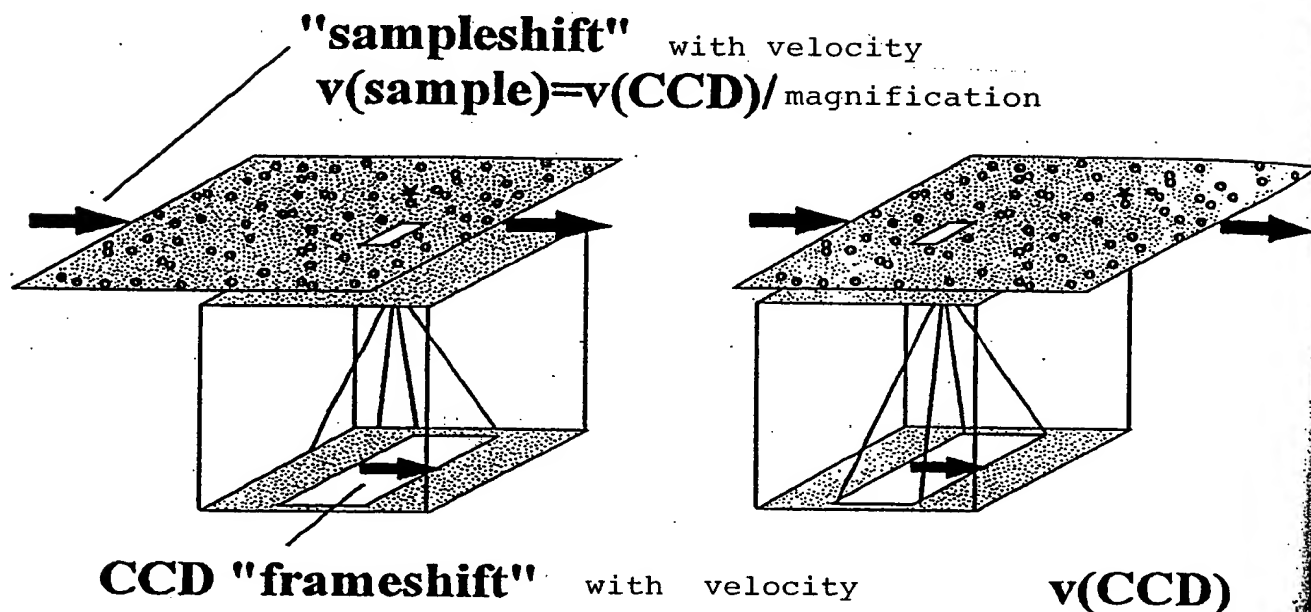
Illumination (continuous, spatially fixed)
either as in Fig. 1, or by emanescent
excitation at total reflection:

Prism
(spatially fixed)

Laser light
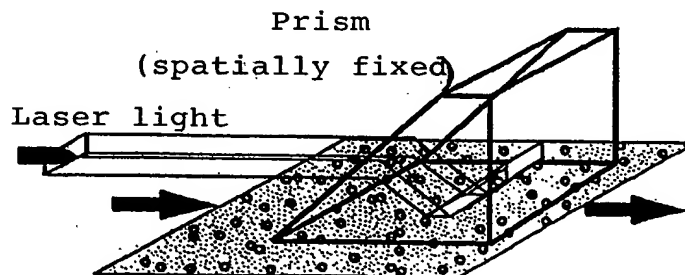
FIG. 4

Screening of units on surfaces

or in multiwell plates

Image of unit sought

(⋆)
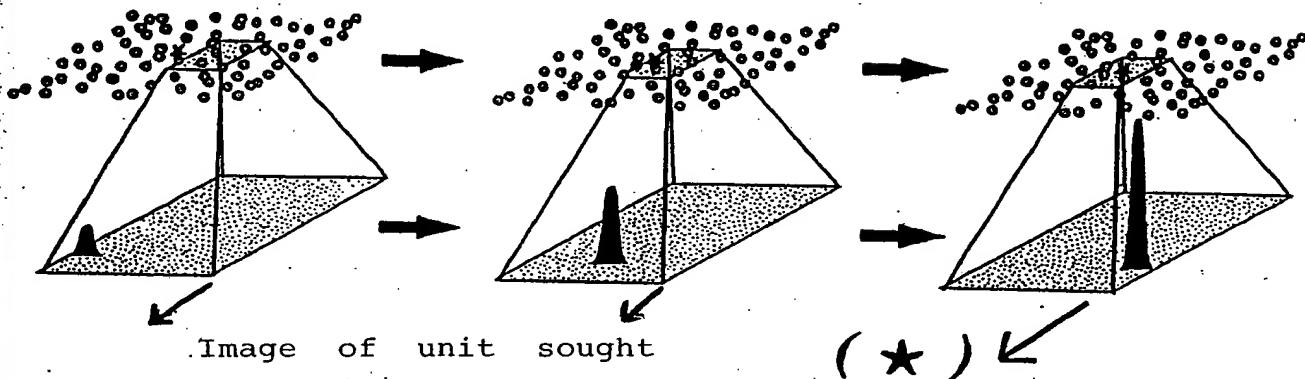
FIG. 5

Screening in laminar flow cell

Image of unit sought (⋆)
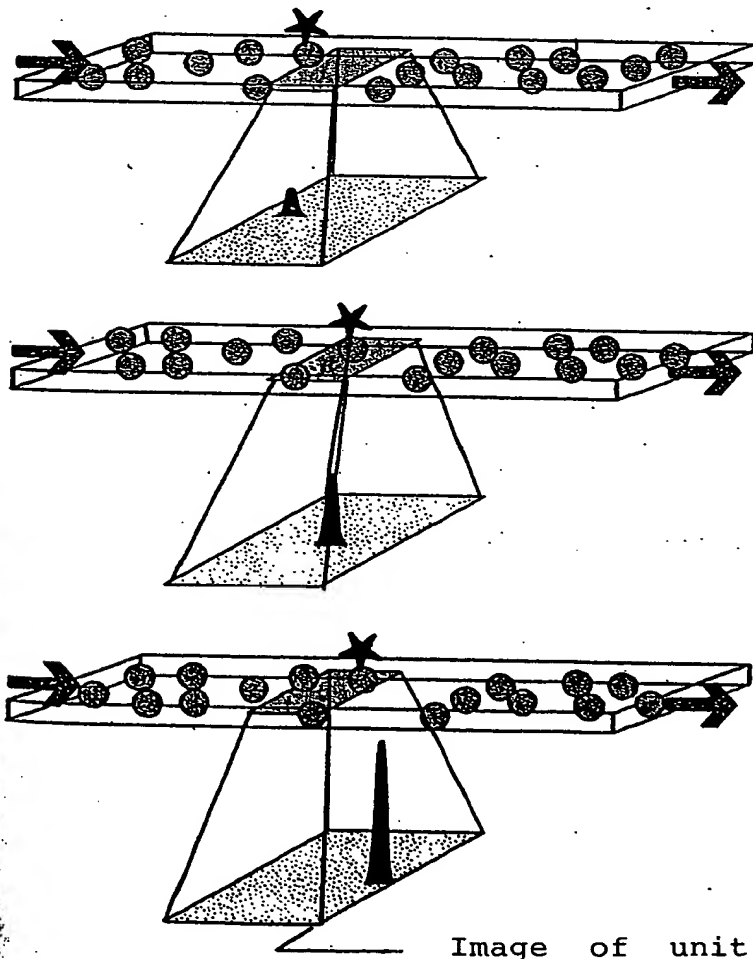
FIG. 6

Screening-time and resolution of units on 1 inch*inch area (e.g. 1 million cells) with single fluorophore sensitivity
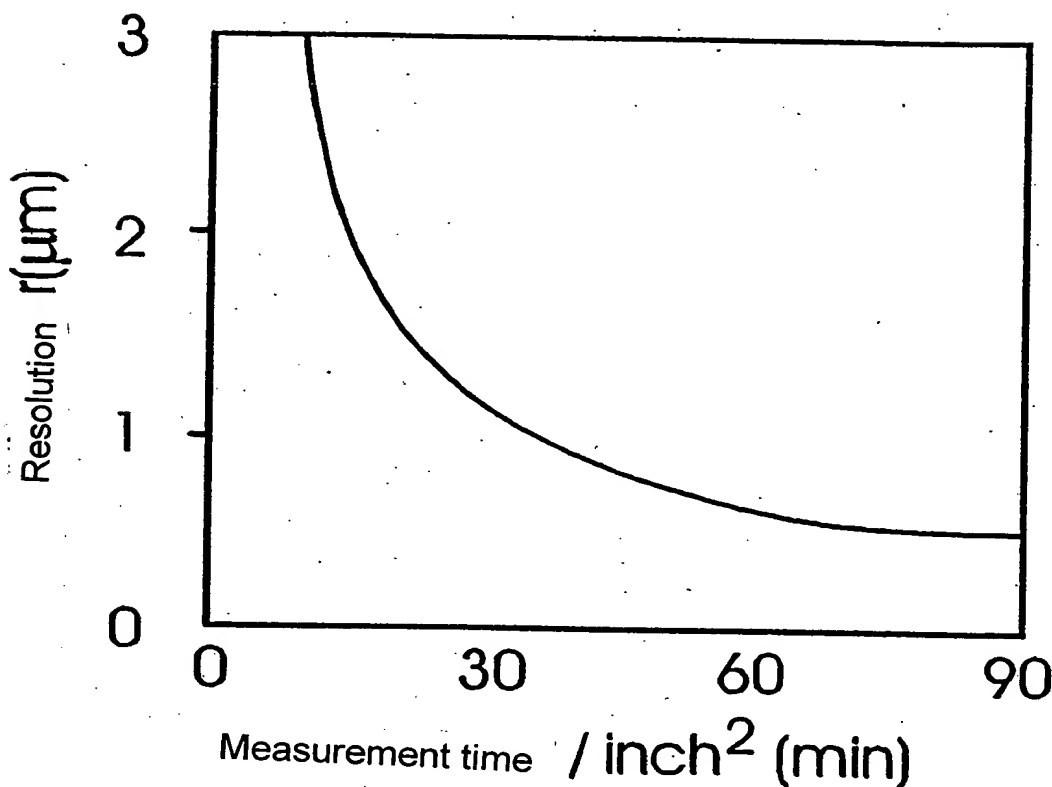
for typical characteristics of CCD cameras
(cooled with liquid N_2): 1024*256 pixel (25*25μm)
"lineshift" rate 10 KHz, readout rate 200 KHz,
readout noise ~ 5 counts, dark noise ~0.
Objective with 63-fold magnification,
illuminated area = area pixelarray/63*63=
400μm*100μm; light intensity kept at 500 counts per
fluorophore.

at 4 sec waiting after each discrete image $\rightarrow \Delta T^{tot} = 4 \sec . \dfrac{1 \text{ inch}^2}{400 \times 100 \ \mu m^2} = 1040 \text{ min}$

FIG. 7

ANALYSIS of DETECTED UNITS
by single fluorophore micriscopy (cf. Fig. 1)
either directly after screening   or after transfer

buffer

buffer

illumination
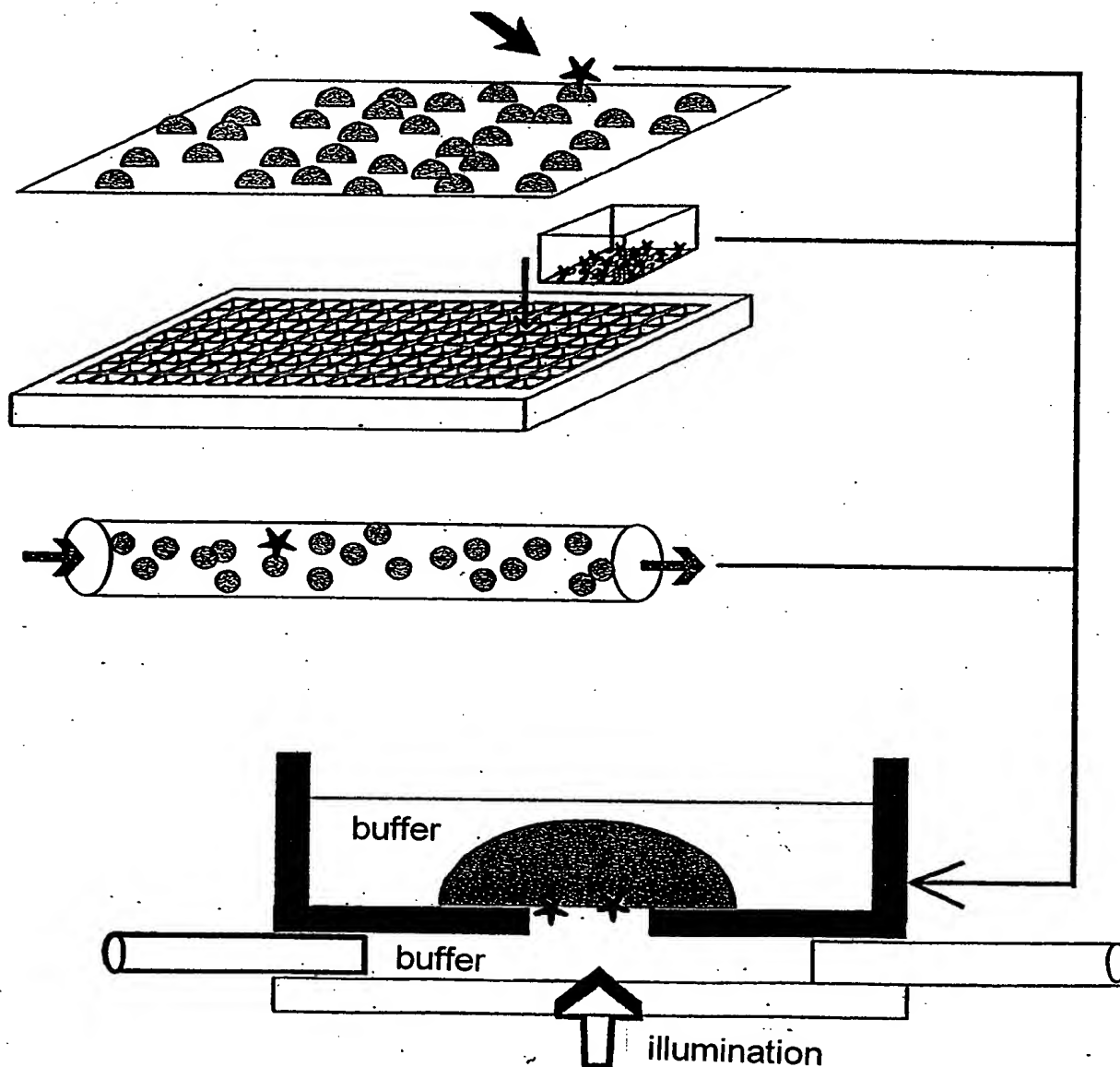
in an analysis cell (flowthrough cell next to screening cell)

FIG. 8

USE of
DONOR-ACCEPTOR ENERGY TRANSFER
for HIGHLY SELECTIVE SCREENING

Cell membrane

Image
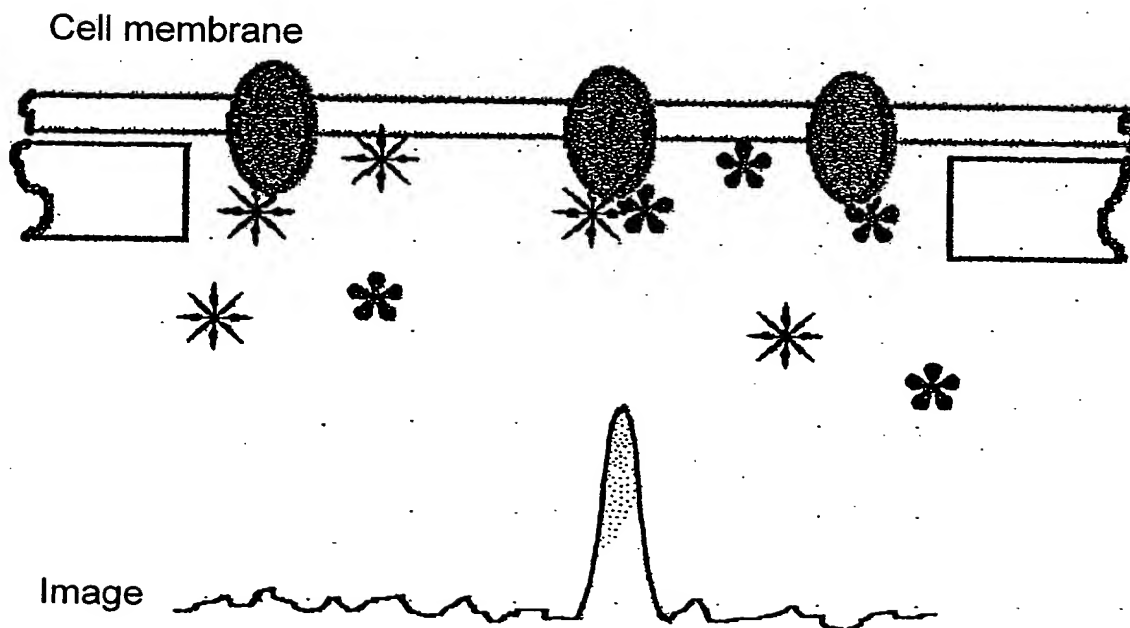
�֍ **DONOR FLUOROPHOR**

✳ ACCEPTOR FLUOROPHORE

Excitation: only donor fluorescence
Collection: only acceptor fluorescence

FIG. 9

1. POSITIONS of LABELLED MOLECULES

Cell membrane

Image
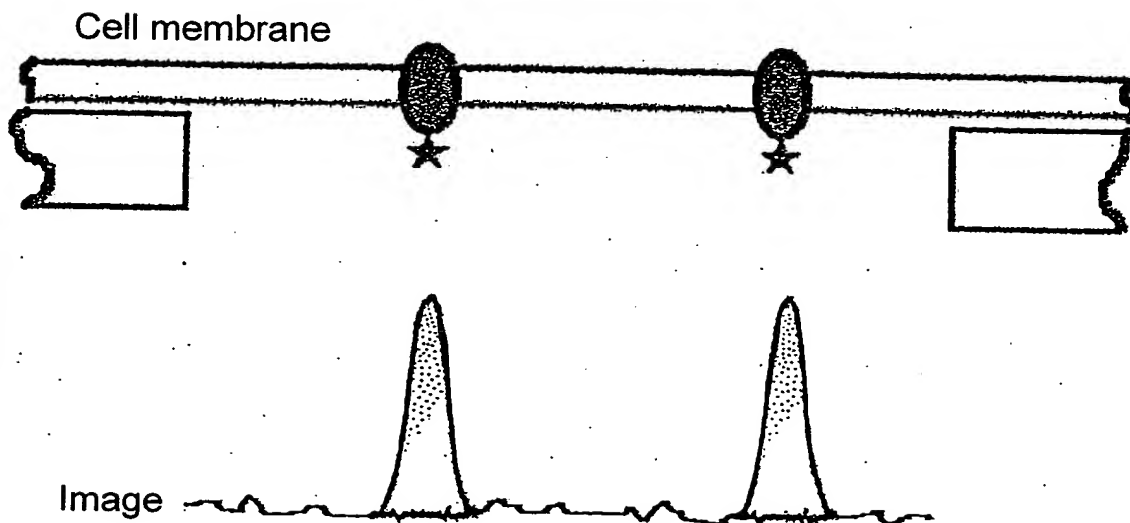
FIG. 10

TEMPORAL TRACING of the MOLECULE POSITIONS

Image

t_1, t_2

immobile

t_2

t_1

mobile
(free diffusion, or
compartment diffusion)
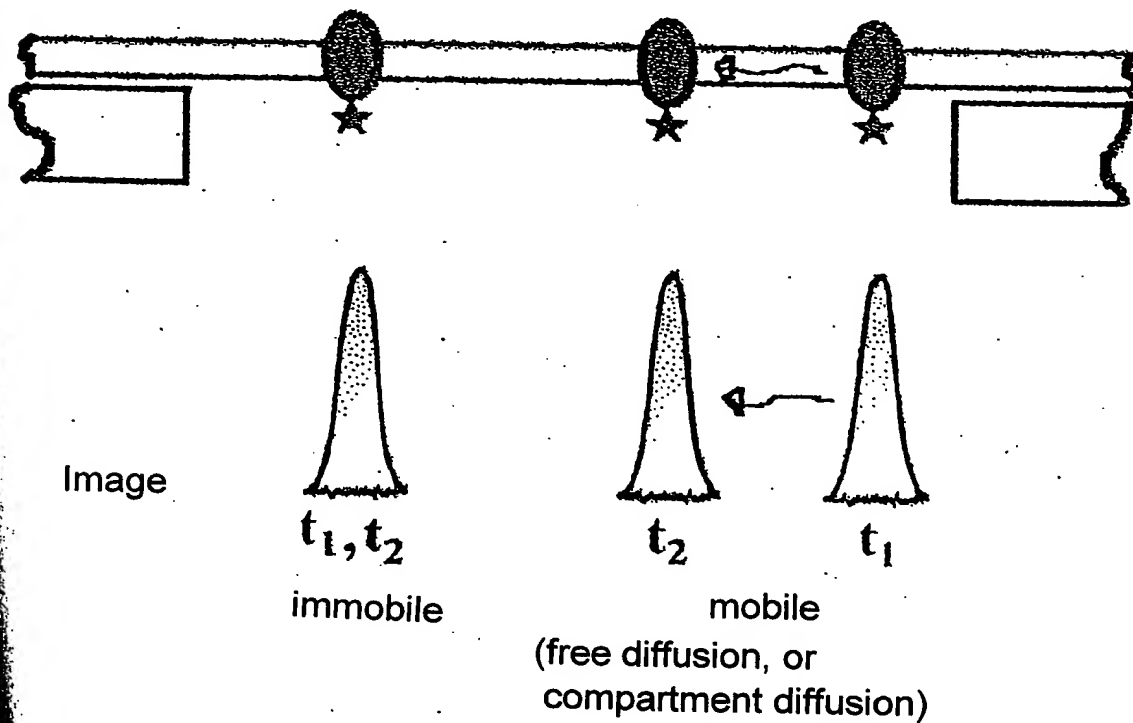
FIG. 11

3. MOLECULAR ASSOCIATION, CO-LOCALISATION, STOICHIOMETRY from SIGNAL QUANTISATION

Cell membrane

t_1

Fluorescence intensity per fluorophore: i_1

t_2

$2*i_1$
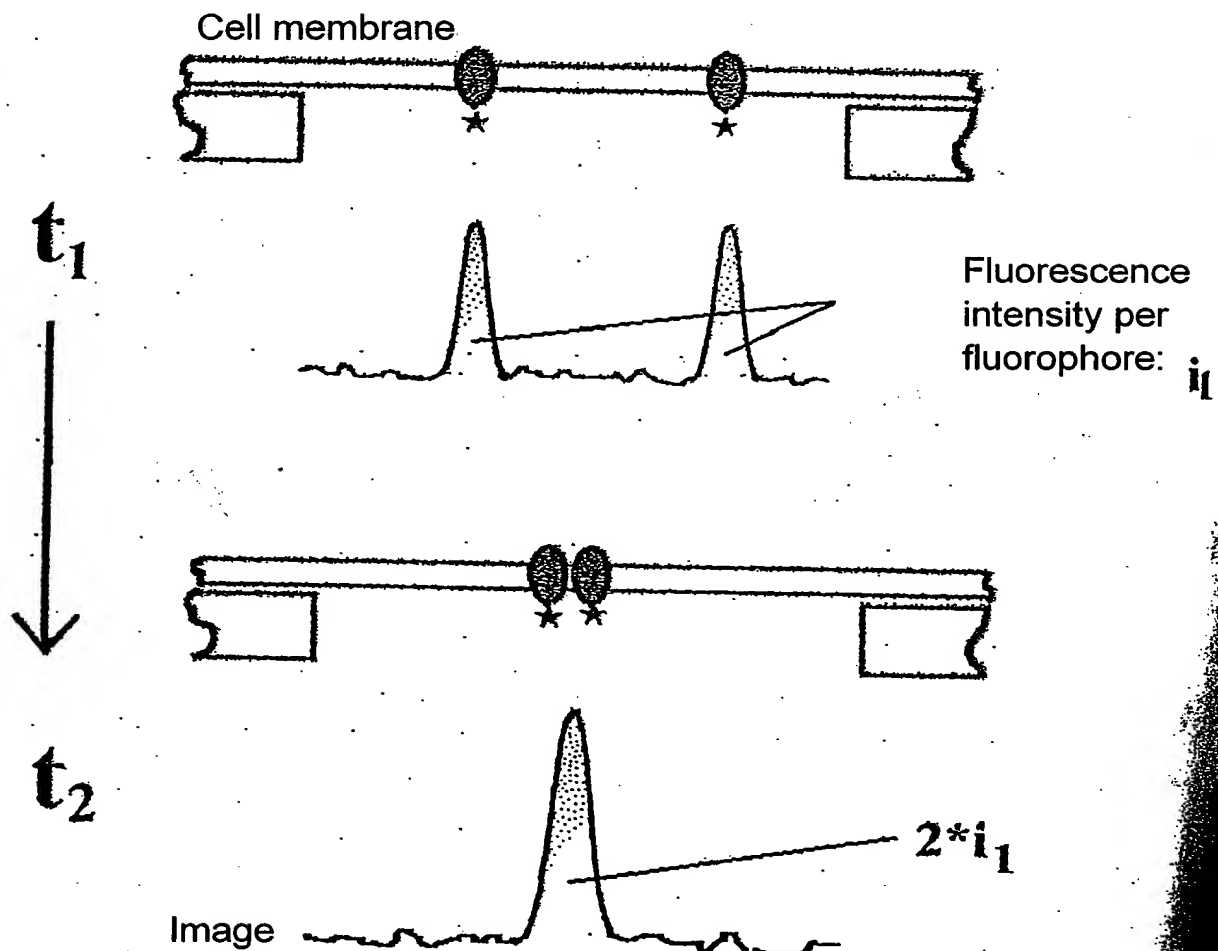
Image

FIG. 12

4. LIGAND BINDING

for
instance

0 of 2
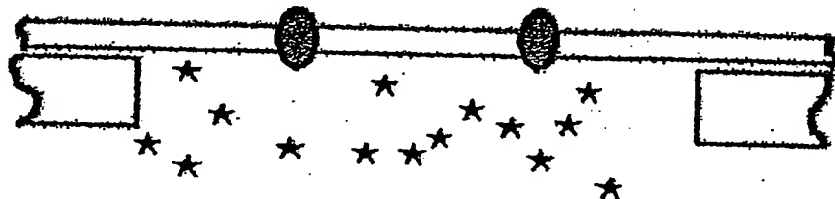
Image
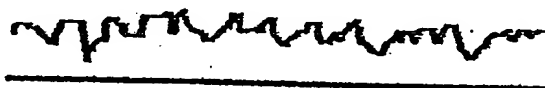
1 of 2
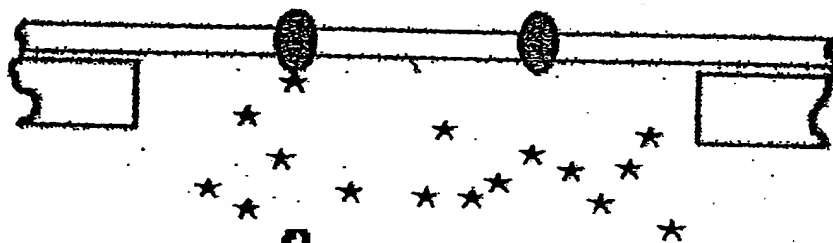
Image
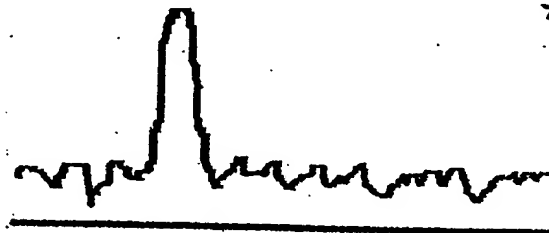
2 of 2
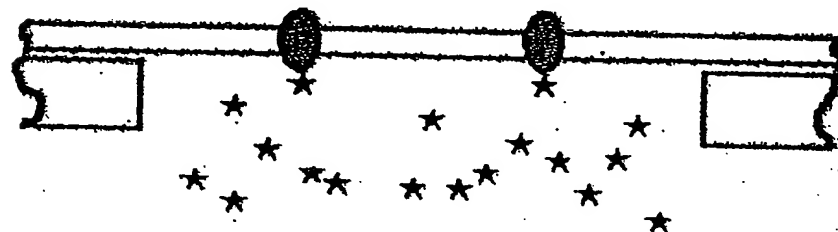
Image

FIG. 13

5. CONFORMATIONAL CHANGE ON SINGLE MOLECULE

031675

FIG. 14

5μm

FIG. 15

1µm

30

0

FIG. 16

5μm

0

25

50

FIG. 17

FIG. 18

FIG. 19

Original

150

0

1µm

FIG. 20A

FIG. 20B